The homeobox transcription factor Hlx modulates yolk sac vascular remodeling in mouse embryos

Running title: Vascular Development in Hlx-null embryos.

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ABSTRACT
OBJECTIVE: The divergent homeobox transcription factor Hlx plays an essential role in visceral organogenesis in mice and has been shown to regulate angiogenic sprouting *in vitro* and in zebrafish embryos. We therefore examined the role of Hlx in vascular development in mouse and avian embryos.
APPROACH AND RESULTS: *In situ* hybridization showed that *Hlx* is expressed in a subset of sprouting blood vessels in postnatal mouse retinas and embryos. *Hlx* expression was conserved in quail embryos and upregulated in blood vessels at the onset of circulation. *In vitro* assays showed that *Hlx* is dynamically regulated by growth factors and shear stress alterations. Pro-angiogenic VEGF induces *Hlx* expression in cultured endothelial cells; whereas signals that induce stalk cell identity lead to a reduction in *Hlx* expression. Hlx was also downregulated in embryos in which flow was ablated, while injection of a starch solution, which increases blood viscosity and therefore shear stress, causes an upregulation in Hlx. Hlx knockdown *in vitro* resulted in a reduction in tip cell marker expression and in reduced angiogenic sprouting but $Hlx^{-/-}$ embryos showed no defect in vascular sprouting at E8.5, E9.5 or E11.5 *in vivo*. Vascular remodeling of the capillary plexus was altered in $Hlx^{-/-}$ embryos, with a modestly enlarged venous plexus and reduction of the arterial plexus.
CONCLUSIONS: Our findings indicate that *Hlx* regulates sprouting *in vitro* but that its role in sprouting is non-essential *in vivo*. We find Hlx is regulated by shear stress and a subtle defect in vascular remodeling is present in knockout embryos.

NON-STANDARD ABBREVIATIONS
Ang2 - Angiopoietin-2
Apln - Apelin
Dll4 - Delta-like 4
Esm1 - Endothelial cell-specific molecule 1
Ets-1 - E26 transformation-specific
HLX - H2.0-like homeobox transcription factor
PDGFB - Platelet-derived growth factor B
UNC5B - Uncoordinated 5 homolog B
VEGF - Vascular Endothelial Growth Factor

INTRODUCTION

Homeobox (Hox) genes are evolutionary conserved genes that are chromosomally clustered in many animal genomes [1]. Hox genes encode transcription factors with a conserved 60 amino acid DNA-binding homeodomain, and are involved in transcriptional regulation of eukaryotic developmental processes, particularly in directing tissue morphogenesis and patterning[2].

The H2.0-like homeobox transcription factor (Hlx) is a diverged, non-clustered homeodomain protein, with a pivotal role in visceral organogenesis and enteric nervous system development[3-6]. In mouse embryos, the highest expression of *Hlx* is detected in the mesenchyme of the developing liver and intestine[4, 6]. Hlx is highly conserved in its gene structure, expression pattern and protein sequence in 9 different species, ranging from fish to avians and mammals[7]; which implies an important evolutionary function. Indeed, deletion of the gene encoding *Hlx* in mice results in embryonic lethality at E15, with intestines that fail to elongate and expand likely due to defects in guidance cues in the enteric nervous system [3, 6]. In addition to defects in intestinal growth, *Hlx*$^{-/-}$ embryos exhibit severe anemia and hypoplasia of the liver (3% of its normal size)[4]. These findings demonstrate important developmental functions of Hlx during visceral mesoderm growth.

HLX is also implicated in human pathology: HLX is overexpressed in patients with acute myeloid leukemia (AML), which induces formation of aberrant progenitor cells with unlimited clonogenicity and blocked differentiation. Inhibition of HLX in leukemic cells reduces proliferation and clonogenicity, identifying HLX as a key regulator in immature hematopoietic and leukemia cells and as a prognostic marker and therapeutic target in AML[8, 9].

In endothelial cells cultured *in vitro*, overexpression of HLX was shown to inhibit endothelial cell sprouting, migration and vessel formation, without affecting proliferation. HLX was shown to robustly upregulate expression of repulsive cell-guidance proteins, such as UNC5B, Plexin-A1, and Semaphorin-3G [10]. It was proposed in this context that HLX may function to balance attractive guidance cues (when stimulated by VEGF) with repulsive vessel guidance cues in order to regulate sprouting in endothelial cells [10]. Additionally, in zebrafish, *Hlx* expression is confined to endothelial cells sprouting from the dorsal aorta into the intersegmental region, and not expressed in adjacent non-angiogenic endothelial cells. Morpholino-mediated knockdown of Hlx led to stunted intersegmental vessel sprouts which failed to connect with neighboring vessels; thus Hlx was proposed to promote/reinforce stalk cell potential in this context [11]. These findings showed that both loss- and gain-of function of Hlx affected vessel sprouting, suggesting important roles of this transcription factor in vessel branching morphogenesis. However, previous studies using genetic loss-of-function models in mice have not addressed the role of Hlx in blood vessel formation.

Here we show that endothelial cells express *Hlx* during development in two amniote models; mouse and quail embryos. The expression is not ubiquitous but labels a subset of endothelial cells. We then investigate the function of Hlx in angiogenic sprouting. We find that Hlx controls the expression of some stalk/tip cells genes and that knockdown of Hlx inhibits sprouting *in vitro*. From our time course in early vascular development, we had observed that Hlx expression was strongest in endothelial cells just after the onset of flow. We therefore use three different models to alter flow and look at Hlx regulation in response to flow. We find that shear stress can upregulate Hlx. We investigated whether a vascular phenotype was present in Hlx-null embryos. We find that the intra-embryonic vasculature forms normally and that sprouting is normal in all tissues investigated at E8.5, E9.5 and E11.5. When we looked at the yolk sac vascular plexus, we found a remodeling defect: the venous plexus is enlarged and the vitelline vein is enlarged, as compared to control. A proportional decrease in arterial diameter is present. Our results show that while Hlx can regulate sprouting *in vitro*, its role in vascular sprouting is non-essential in mouse embryos *in vivo*. In contrast, we find that Hlx is upregulated by flow. We also find that Hlx ablation affects vascular remodeling.

MATERIALS AND METHODS

Materials and Methods are available as online supplemental material.

RESULTS

Hlx is dynamically regulated in developing blood vessels in mice and quail embryos

Hlx was previously described as a gene upregulated by VEGF and suppressed by Notch signaling in endothelial cells *in vitro* and in zebrafish embryos [10, 11]. Consistent with these results, we found that compared to wildtype controls, *Hlx* expression was increased (3.5 fold) in microarrays from Dll4$^{+/-}$ retinal endothelial cells, which present an excessive sprouting phenotype[12]. Furthermore, *in situ* hybridization with an *Hlx* antisense riboprobe on postnatal day 5 (P5) mouse retinas showed *Hlx* expression in a subset of tip cells, confirming the microarray results and suggesting a possible function for *Hlx* in sprouting angiogenesis (Figure 1A,B, red and blue arrowheads).

Examination of *Hlx* expression in E10.5 mouse embryos revealed strongly labeled splanchnopleural visceral mesoderm associated with the developing lung and liver, while sense probes showed no specific signal (Figure1C,D). *Hlx* expression was absent in the heart, as well as in the endothelium of the larger vascular structures such as the cardinal vein and the dorsal aorta (Figure 1E). *Hlx* expression was present in vessels branching off the aorta (Figure 1E, black arrowhead) and in the perineural vascular plexus (Figure 1F, arrows). Within the neural epithelium, we failed to detect any *Hlx* staining of endothelial cells (Figure1G,H, green arrowheads). In E12.5 embryos, *Hlx* expression became downregulated in the liver, but remained enriched in splanchnic mesoderm and was also found in the developing sclerotome and neural tube, with strongest expression in the roof plate (data not shown). A subset of smaller blood vessels remained positive for *Hlx* expression in the perineural vascular plexus surrounding the neural epithelium and in the dermis. Capillaries inside the neural epithelium however, did not stain positive for *Hlx* expression at E10.5 or E12.5. Together, these expression studies confirm previous reports demonstrating preferential *Hlx* expression in developing visceral mesoderm of mouse embryos [3, 4, 6, 7, 13] and show that a subset of sprouting vessels in the mouse retina and in the embryo, respectively, express *Hlx*.

To assess *Hlx* expression in another developing amniotic model, we performed whole mount *in situ* analysis on developing quail embryos. Consistent with our findings in mice, we found *Hlx* to be enriched in the splanchnic mesoderm, prior to and during formation of the intestine and liver (Figure 2A,B). In addition to the splanchnic mesoderm, *Hlx* is also detected in the branchial arches but not in the heart (Figure 2A-B, arrowheads). In the embryo proper, Hlx expression was absent in endothelial cells lining the heart and the dorsal aorta, consistent with previous reports [7].

We noticed a significant level of *Hlx* expression in the capillary plexus of the yolk sac. We chose 3 specific stages (8 somites, 14 somites and 20 somites) at which to look at vascular expression. Vascular development begins in the extra-embryonic capillary plexus at 0 to 5 somites in quail embryos, when blood islands establish the primary components and set the outline of the blood vessel network. Once the initial plexus is formed *de novo*, the heart begins to beat and the plexus undergoes vascular remodeling. Erythroblasts enter the circulation between 11-13 somites. The first morphologically distinct arteries and veins are present by approximately 20 somites (mid-vascular remodeling stage), and the mature architecture of the vascular network is attained at 4 days, or Hamburger Hamilton stage 23.

In the quail extra-embryonic region, we detected *Hlx* in mesodermal and endothelial cells of the capillary plexus at all stages investigated (Figure 2C). The expression pattern of *Hlx* was ubiquitous throughout the plexus and did not show arterial-venous specificity (data not shown). Sense probes did not show any specific signal (Figure 2D). To quantify Hlx expression levels in endothelial cells, we isolated endothelial cells using magnetic beads (see Methods). Our data

shows that at the protein level, Hlx is robustly upregulated in endothelial cells at the 14-somite stage (Figure 2E). Expression then plateaus at mid- vascular remodeling stage (20 somites) and mature remodeled stage (4 days, or Hamburger Hamilton stage 23). Therefore, Hlx is upregulated in the capillary plexus at the 14-somite stage, which coincides with the onset of blood circulation.

Hlx regulates endothelial sprouting in vitro

To examine Hlx regulation and function in sprouting angiogenesis, we tested *Hlx* expression levels in HUVECs stimulated with VEGF or sDLL4 and BMP9, which promote tip and stalk cell fate, respectively. A 24-hour stimulation of VEGF induced an eight-fold increase in the expression of *Hlx* (Figure 3A), while sDll4 and BMP9 both led to a reduction in *Hlx* expression (Figure 3B). We then asked whether Hlx loss-of-function affects downstream expression of tip and stalk cell genes. siRNA-mediated knockdown of *Hlx* (Figure 3C, 75% knockdown efficiency) reduced expression of some tip cell-enriched genes including *Esm1* and *Dll4* but expression of *Unc5B*, *Nidogen-2, Apln*, *Ang2, PDGFB* and *uPAR* was not affected by *Hlx* knockdown (Figure 3D). Furthermore, *Hlx* knockdown failed to increase expression of stalk cell genes *Ets1, Tie2* and *Jag1* (Figure 3E). These data suggest that *Hlx* affects expression of some, but not all, tip cell genes. To further investigate the role of Hlx in sprouting angiogenesis, we used an *in vitro* sprouting assay with HUVECs embedded in 3D-fibrin gels [14]. siRNA-mediated knockdown of *Hlx* significantly reduced VEGF-induced endothelial sprouting (Figure 3F). Taken together, these results suggest that Hlx expression regulates the expression of only a few tip cell genes, but that it does influence sprouting angiogenesis *in vitro*.

Hlx expression is regulated by flow and shear stress levels *in vivo* and *in vitro*

Given that we saw a significant increase in Hlx expression in endothelial cells at the onset of circulation, we next investigated whether blood flow regulated Hlx expression. To test this, we performed loss-of-flow (LOF) and gain-of-flow (GOF) experiments in quail embryos. For LOF, we ablated flow by snipping the inlet to the heart at 8 somites and allowed the embryo to develop until 18 somites, which results in embryos with no cardiac output and arrested vascular remodeling[15]. The expression of Hlx in the absence of flow was then determined by Western blot analysis of whole embryo lysates. We found that Hlx expression is significantly decreased in embryos lacking blood flow when compared to stage-matched controls (Figure 4A).

Blood flow creates a mechanical force on endothelial cells called shear stress, which is a function of fluid viscosity, flow rate and vessel diameter. Thus, by increasing the viscosity of blood one can increase the shear stress in the circulation without affecting flow velocity. Therefore, for the GOF experiments, we increased shear stress levels in the circulation of embryos by microinjecting a viscous starch solution at the 14-somite stage, using a method we previously developed [16]. Hlx protein expression was analyzed 6 hours post injection, and found to be significantly increased in embryos with increased shear stress (Figure 4B). Densitometric analysis confirms significant up-regulation of Hlx in embryos in the vascular system in which we altered blood viscosity. These data suggest that Hlx expression is not only endogenously upregulated at the onset of blood circulation, but also affected by manipulating patterns of flow in the embryo.

We further investigated the possibility that changes in shear stress can affect *Hlx* expression in an *in vitro* system. We exposed cultured human abdominal aortic endothelial cells (HAAEC) to flow using a parallel plate flow chamber. HAAEC were seeded on a microscope slide, placed in a parallel plate flow chamber and exposed to low levels of laminar flow (5 dyn/cm^2) for 1 hour. These levels are similar to the level of shear stress present at the onset of circulation [17]. Our data shows that in cultured endothelial cells, *Hlx* RNA expression is significantly upregulated by steady embryonic-like flow compared to control static conditions

(Figure 4C). Western blot confirms that Hlx protein expression is upregulated in endothelial cells stimulated with embryonic laminar flow for 6 hours (Figure 4C).

Hlx$^{-/-}$ embryos show normal angiogenic sprouting but defective yolk sac vascular remodeling

To determine if Hlx function was required for angiogenic sprouting and/or yolk sac vascular remodeling *in vivo*, we next examined vascular development in *Hlx*$^{-/-}$ mouse embryos [4]. Intraembryonic vascular development appeared to occur normally in these embryos. At E8.5, the dorsal aorta and heart had formed normally (Figure 5A). At E9.5, intersomitic vessels sprout from the dorsal aorta and fuse to form the dorsal vein. No difference was observed between control or knockout embryos (Figure 5B). Formation of the branchial arch arteries was also phenotypically normal as was cranial vascularization. Staining for Endomucin also did not reveal any differences in the vasculature of the embryo at E9.5 between heterozygous and knockout littermates (Figure 5C).

Though intra-embryonic sprouting angiogenesis was normal, we next looked at vascular remodeling in the yolk sac of *Hlx*-null embryos. The yolk sac capillary plexus forms as an immature plexus that undergoes remodeling between E8.5 and E9.5. We found no defects in the capillary plexus of Hlx$^{-/-}$ embryos at E8.5, before the onset of flow (data not shown). At E9.5, Hlx$^{-/-}$ yolk sacs had undergone vascular remodeling and were not grossly affected by ablation of Hlx as compared to control (Figure 5D). However, closer inspection of the remodeled E9.5 vascular plexus showed defects in the conformation of the arterial and venous plexus. To quantify the morphological differences in the *Hlx*$^{+/-}$ and *Hlx*$^{-/-}$ vascular plexus, we measured lumen diameter of the veins and arteries; the surface area with arterial versus venous morphology; the alignment of arterial endothelial cells with the direction of blood flow; the endothelial cell shape index and the density of branchpoints in the arterial and venous plexus. Among these morphometric measurements, the surface area of the *Hlx*$^{-/-}$ venous plexus and the diameter of the vitelline vein and artery were significantly different when compared to controls. Specifically, the venous plexus occupies a larger percentage of the vascularized yolk sac than in wild-type embryos (Figure 5D, E). The vitelline artery tree is reduced in size and the vitelline vein is increased in diameter (Figure 5E). Additionally, we investigated arterial-venous identity and found normal expression of Cx40, an arterial-specific marker, in the Hlx$^{-/-}$ vascular plexus (Figure 5F), suggesting that induction of arterial gene expression is unaffected.

We next evaluated Hlx deficient embryos at E11.5 through hematoxylin and eosin staining on sections. As previously reported[4], we found that the liver completely failed to form in Hlx-null embryos (Figure 6A,B, arrow). Though the intestine formed, its development was significantly retarded (Figure 6A,B).

Examination of vascular development at this stage by CD31 staining of transverse sections through the embryo showed no defect in the dorsal aorta or its branches (Figure 6C). Smooth muscle cells were present around the dorsal aorta and no differences were observed in the thickness of the smooth muscle cell layer (Figure 6D). On longitudinal sections through the embryo, both intersomitic arteries and veins appeared normal (Figure 6E). The embryonic hindbrain is vascularized by angiogenic sprouting between E10.5 and E11.5, therefore we measured vascular density at E11.5 in *Hlx*$^{+/+}$and *Hlx*$^{-/-}$ hindbrain wholemounts (Figure 6F). Quantification of branchpoint density showed no difference between wild-type embryos and *Hlx*$^{-/-}$ embryos. Therefore, the remodeling defect that occurred in the yolk sac at E.9.5 did not lead to detectable intraembryonic vascular defects by E11.5, suggesting that Hlx modulates vessel remodeling but is dispensable for angiogenic sprouting in mice.

DISCUSSION

In zebrafish embryos, *Hlx* expression has been shown to be dynamically regulated during sprouting angiogenesis, with initial tip-cell selective expression that subsequently becomes

restricted to stalk cells and is absent from other embryonic endothelial cells [11]. We show here that Hlx is also expressed in subsets of sprouting vessels in mouse embryos *in vivo*, including tip cells in the developing retina. We had previously performed microarray comparison between wildtype and Dll4$^{+/-}$ retinal endothelial cells and found Hlx to be upregulated in the Dll4$^{+/-}$ population. Furthermore, we show that in cultured human endothelial cells, Notch activation by Dll4 decreases Hlx expression, while VEGF increases it, as reported previously [10]. These data, together with other reports[10, 11], show that Hlx regulation by VEGF and Notch signaling is conserved between zebrafish, mouse and human endothelial cells. Overexpression of Hlx in cultured endothelial cells had previously been shown to inhibit angiogenic sprouting through upregulation of guidance receptors including the repulsive Netrin receptor UNC5B [10, 18]. Here we show that knockdown of Hlx also inhibits endothelial sprouting *in vitro*, indicating that both loss- and gain of Hlx function affect in vitro sprouting. In line with these observations, Hlx knockdown in zebrafish embryos led to decreased intersomitic vessel formation, indicating functional requirement of Hlx for angiogenic sprouting in this species *in vivo* [11]. In contrast, we show here that sprouting angiogenesis occurs normally in mouse embryos deficient for Hlx. Hlx$^{-/-}$ embryos die during mid-gestation because of severe anemia due to defective liver development [4]. Analysis of vessel development in embryos between E8.5 and E11.5 failed to reveal any obvious defects in formation of major vessels such as the dorsal aorta and cardinal vein or their side branches. Thus, in contrast to the *in vitro* setting and zebrafish *in vivo* data[10, 11], loss of Hlx in the mouse embryo does not affect sprouting angiogenesis. This is in agreement with the *in situ* hybridization results, which show that Hlx is expressed in only a subset of sprouting vessels. Furthermore, our *in vitro* experiments show that most tip and stalk cell genes are unaffected by Hlx knockdown. Therefore, we have been unable to confirm a role for Hlx in sprouting angiogenesis in the mouse embryo. Although we cannot exclude the possibility that minor phenotypes may have been missed, our results indicate that Hlx is dispensable for sprouting in mouse embryos. Inducible, endothelial specific Hlx knockout may reveal a role for Hlx in sprouting in other tissue beds and/or at later stages of development or in pathology.

In addition to expression in tip cells and sprouting vessels, we found that endothelial-specific Hlx expression was increased at the onset of circulation. We confirmed that blood flow and more specifically shear stress was responsible for the increase in Hlx expression using 3 different models: 1) by ablating flow in the embryo, 2) by increasing shear stress in the embryonic circulation and 3) by exposing human endothelial cells to shear stress *in vitro*. All three models confirmed that Hlx is upregulated in response to steady laminar type flow. *In vivo*, we observed a yolk sac remodeling defect after the onset of extraembryonic blood flow, suggesting that blood flow is a physiological signal for Hlx regulation and that Hlx is required for vessel remodeling. The defect in remodeling may be a direct cause of the defective shear-induced signaling pathways, but this remains to be definitively proven. In the intestine, expression of Hlx by mesenchymal cells has been shown to direct neural crest cell migration[3]. Given that we observe Hlx expression in mesodermal cells of the yolk sac, we cannot exclude the possibility that factors expressed by non-endothelial cells are driving the remodeling phenotype. Though the relative balance of arteries and veins is altered in the knockout embryos, it is important to note that expression of arterial-specific gene is unaffected in the Hlx$^{-/-}$ vascular plexus, as shown by Cx40 staining. Signals from shear stress are transduced through the VEGF receptor VEGFR2 [19], suggesting that Hlx may be induced downstream of VEGFR2 signaling in both angiogenic sprouts and perfused embryonic blood vessels. Whether shear-induced expression of Hlx has an effect on angiogenic sprouting, however, is unclear.

Using adenoviral constructs to upregulate recombinant Hlx, Testori *et al*. showed that Hlx expression induces an upregulation of Notch targets including Unc5B, PlexinA1, Sema3G and Hes1 [10]. Unc5B [20], Sema3G [21] and Hes1 [22] all display arterial specific expression patterns. Interestingly, the endothelial specific double-mutant of Hes1/Hes5 shows a very comparable phenotype to the Hlx$^{-/-}$ mutants [22]. The double mutants exhibit a defect in vascular remodeling

such that the diameter of vessels branching off the main carotid artery is decreased, but no change in the diameter of the major arteries themselves is observed. The authors did not investigate the venous vasculature. Given that Hlx ablation results in a similar phenotype to Hes1/5 ablation, we propose that Hlx is upstream of Hes1 in the process of vascular remodeling.

The promoter of Hlx shows a significant number of shear-response elements (SSRE) previously shown to regulate shear response in gene promoters. *In silico* analysis of the aligned mouse and human *Hlx* promoters revealed the presence of 28 different transcription factor binding sites and cis-acting elements conserved on the promoter, which have been previously shown to regulate shear response in other gene promoters. It is important to note that the transcriptional responsiveness to shear stress is determined by a combination of SSRE rather than by a single SSRE. Most notably, two inverted CCAAT motifs are found in the *Hlx* promoter, which were previously identified to be functionally important for *Hlx* expression [23]. The CCAAT motif binds CCAAT-enhancer-binding proteins (C-EBP), and has been identified as a positive and robust SSRE [24]. Furthermore, two KLF2 binding sites (2 CACCC boxes, located within 50bp of start site) are located proximal to the mouse and human *Hlx* start site. Expression of KLF2 regulates many shear-expressed genes [25, 26]. Given the conserved presence of multiple shear stress response elements on the mouse and human *Hlx* promoters, including the well-characterized KLF2, we propose that Hlx is upregulated in response to shear stress by shear-sensitive transcription factors; and that it is a downstream effector of mechanotransduction rather than a primary transcription factor required for mechanosensitive gene induction.

Though our finding shows that Hlx is required for normal yolk sac vascular remodeling at E9.5, this defect does not lead to apparent major intra-embryonic vessel remodeling defects at E11.5. We did not pursue analysis of potential flow-induced phenotypes later in development since flow-induced defects cannot be differentiated from defects in hematopoiesis seen in $Hlx^{-/-}$ embryos. Primitive hematopoiesis, which begins in the yolk sac at E7.5, migrates to the embryonic liver at E10.5, where the secondary site of hematopoeisis takes place. The Hlx-null embryos fail to form a liver and are anemic by E13.5 [4], inevitably resulting in a decreased hematocrit due to reduced hematopoietic cell population. Shear stress is a function of both rate of blood flow but also the viscosity of the blood. The hematocrit is the primary determinant of blood viscosity. As such, as the $Hlx^{-/-}$ embryos become anemic by E13.5, the shear stress levels also decrease. In order to study the effects of Hlx on flow-induced remodeling in the vasculature independently of liver defects, an endothelial-specific deletion of Hlx would be necessary.

Taken together, our results show that *Hlx* expression is conserved in a subset of developing blood vessels in mouse and avian embryos and indicate that its expression in developing blood vessels is dynamically regulated. Our *in vitro* results show that Hlx expression can be controlled both by signals for sprouting and by shear stress. Our *in vivo* results show that the role of Hlx in sprouting is non-essential. Hlx-null embryos do show a defect in vascular remodeling in which patterning is abnormal.

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DISCLOSURES

The authors have nothing to disclose.

SIGNIFICANCE

Identifying the genes that control sprouting angiogenesis are of vital importance to targeting the angiogenic process. Certain signals induce endothelial cells to become “leader cell”, or tip cells, during sprouting whereas other signals induce cells to become trailing cells or “stalk cells”. Hlx has recently been suggested to be an important regulator of stalk cell identity and to balance attractive guidance cues during sprouting. Our *in vitro* results agree with this role. *In vivo*, however, Hlx null embryos lack a sprouting phenotype. We did, however, find that Hlx is upregulated *in vivo* at the stage where blood flow begins. Using gain- and loss-of-function experiments, we found that Hlx expression is regulated by flow. When we investigate vascular remodeling in the embryo, we find abnormal patterning. The shear-induced expression of Hlx in endothelial cells is completely novel, which has previously only been associated only with sprouting angiogenesis.

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Figure 1 –*Hlx* is expressed in some but not all sprouting vessels. *Hlx* expression in P5 mouse retinas is enriched in a subset of tip cells (tip cells positive for Hlx; red arrowheads and tip cells negative for Hlx; blue arrowheads, A-B). Sections of E10.5 embryos shows expression in the splanchnic mesoderm (C) associated with the developing liver (Li) and lung (L) but absent from heart (Hrt). Sense probes were produced as control (D). Expression is absent in the endothelium of the aorta (Ao, E) but present in vessels sprouting off the aorta (black arrowhead). The strongest expression is seen in the developing intestine (Int, E). *Hlx* is also expressed in the vascular plexus surrounding the neural tube (Nt) (F, arrows) but absent in endothelial cells sprouting into the neural epithelium (G, H, green arrowheads). Sections in G,H are higher magnifications of a region of neural epithelium equivalent to the region boxed in F. Sections were counterstained with anti-Coll IV antibody (G). Scale bars: 50 μm (A, B) ; 1000 μm (C, D); 50 μm (E, F); 20 μm (G,H).

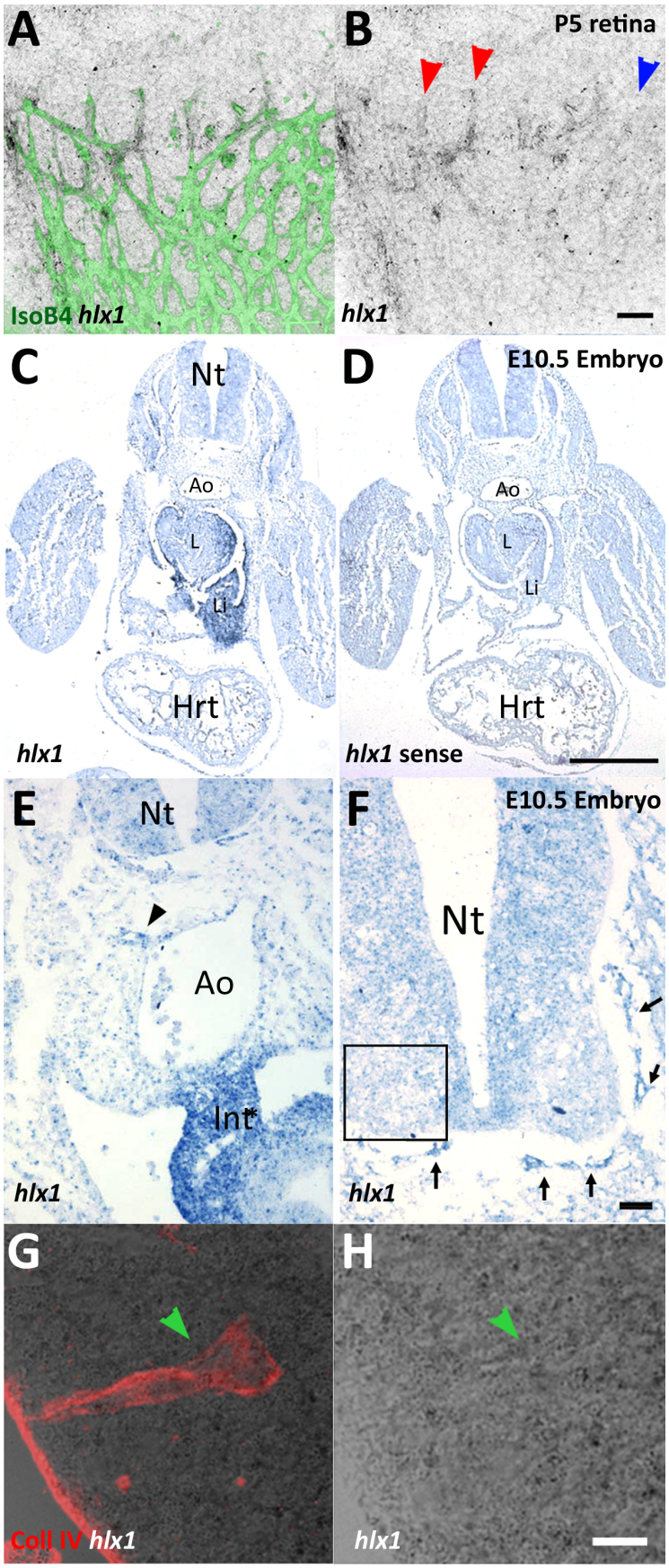
Figure 2 – *Hlx* is upregulated at the onset of blood flow. *In situ* analysis of 14 (A) and 20 somite-stage (B) quail embryo shows intraembryonic expression is confined to the splanchnic mesoderm and the branchial arches (arrowheads). No expression in the dorsal aorta or heart is observed. Wholemount *in situ* analysis shows that *Hlx* expression is observed in the yolk sac at 8 somites (before blood circulation), at 14 somites (onset of circulation), and at 20 somites (C). Sense probe was produced as control (D). Endothelial cells were isolated from pools of whole quail embryo lysates and analyzed for endogenous Hlx protein expression (E). Hlx protein expression in endothelial cells peaks at 14 somites following the onset of blood flow in the embryo; and plateaus at 20 somites and at 4 days of development. Protein loading was assessed by Coomassie staining (bottom panel). Densitometric analysis confirms significant up-regulation of Hlx at 14 somites, when normalized to 7 somites (n=3 samples per stage, between 3 and 25 embryos per sample depending on stage). All values are mean ± SEM. *$p < 0.05$; **$p < 0.01$; two-tailed Student's t-test. Scale bars: 500 μm (A, B); 200 μm (C, D).

Figure 3 – Hlx knockdown regulates sprouting *in vitro*. Stimulation of endothelial cells with VEGF caused a significant increase in *Hlx* expression (n=8, A) whereas stimulation with BMP9 and Dll4 significantly decreased *Hlx* mRNA levels (n=3, B). Using siRNA against Hlx, we achieve a 75% knockdown (n=5, C). Two of eight tip cell markers were significantly downregulated by knockdown of *Hlx* (n=3 to 5, D). Of the three stalk cell markers investigated, only *jagged1* expression was increased, albeit not significantly (n=3, E). Knockdown of *Hlx* also caused a 50% reduction in the ability of endothelial cells to form tubes in fibrin gel (n=3, F). All values are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; two-tailed Student's t-test. Scale bars: 100 μm (F).

Figure 4 – Shear stress regulates Hlx expression levels. For the loss-of function experiments, flow was ablated at 8 somites in quail embryos and Hlx protein levels were assessed at 18 somites. Hlx expression is downregulated when blood flow is ablated, as compared to age-matched controls (A, n=3, 3-6 embryos per sample). Protein loading was assessed by Coomassie staining (bottom panel). For the gain of function experiments, quail embryos were injected with a starch solution at 14 somites and Hlx protein levels were assessed at 22 somites. Hlx is upregulated when blood viscosity is increased compared to age-matched controls (B, n=3 with 3-6 embryos per sample). Western Blot analysis of human endothelial cells exposed to laminar flow for 6 hours (5 dyn/cm^2) confirms that Hlx protein expression is upregulated by shear stress (n=3, C). *Hlx* RNA levels were also increased by simulating HAAECs to laminar flow for 1h (n=6). All values are mean ± SEM. ∗$p < 0.05$; ∗∗∗$p < 0.001$; two-tailed Student's t-test.

Figure 5 – Remodeling defects are present in the Hlx$^{-/-}$ embryo. CD31 staining of endothelial cells at E8.5 shows no detectable difference between Hlx$^{+/+}$ and Hlx$^{-/-}$ embryos (A). At E9.5, Hlx$^{-/-}$ embryo proper also shows normal vascular development and intersomitic sprouting compared to Hlx$^{+/-}$ embryos (B). Higher magnification images of embryos stained for Endomucin also failed to identify any defects in intersomitic sprouting (C). In the capillary plexus of the yolk sac, the venous plexus (V) occupies a slightly larger fraction of the vascular plexus than the arterial region (A) in Hlx$^{-/-}$ embryos as compared to heterozygous littermates (D). Quantification of the surface area with arterial versus venous morphology confirms these differences (n=9 for Hlx$^{+/-}$ and n=5 for Hlx$^{-/-}$, E). The diameter of the vitelline artery and vein were also significantly different between Hlx$^{+/-}$ and Hlx$^{-/-}$ embryos (E). Staining for Cx40 showed that arterial differentiation was normal (F). Scale bars: 1000 μm (A, B, D); 200 μm (C) and 300 μm (F). All values are mean ± SEM. $*p < 0.05$; $**p < 0.01$; two-tailed Student's t-test.

Figure 6 – Vascular development is normal in Hlx$^{-/-}$ embryos at E11.5. The lung (Lu), liver (Li) and intestine (In) form normally in wild-type embryos (A), but in Hlx$^{-/-}$ embryos the liver fails to form (arrow) and the intestine is abnormal (B). CD31 staining on sections shows that the aorta (Ao) and cardinal vein (CV) are similar in wild type and knock-out embryos (C). Alpha-smooth actin staining also showed normal development of smooth muscle around cardinal vein, aorta and aorta branches (arrows, D). Both intersomitic arteries (black arrows) and veins (red arrows) are normal (E). Quantification of branch points in isolectinB4-stained E11.5 hindbrain wholemounts showed no change in density in knock-out embryos as compared to wild-type littermate controls (n = 4-5 embryos, F). All values are mean ± SEM. Scale bars: 500 μm (A-B), 100 μm (C,D,E) and 200 μm (F).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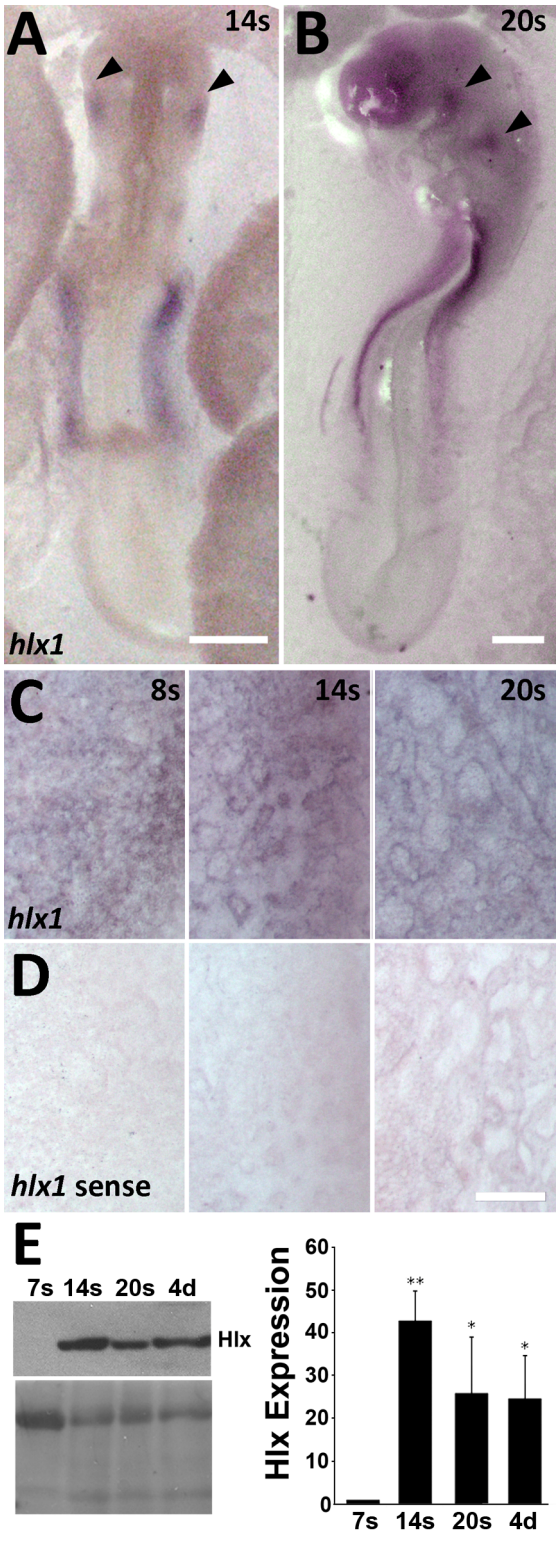
IsoB4 hlx1
B
P5 retina
hlx1
C
Nt
Ao
L
Li
Hrt
hlx1
D
E10.5 Embryo
Ao
L
Li
Hrt
hlx1 sense
E
Nt
Ao
Int*
hlx1
F
E10.5 Embryo
Nt
hlx1
G
Coll IV hlx1
H
hlx1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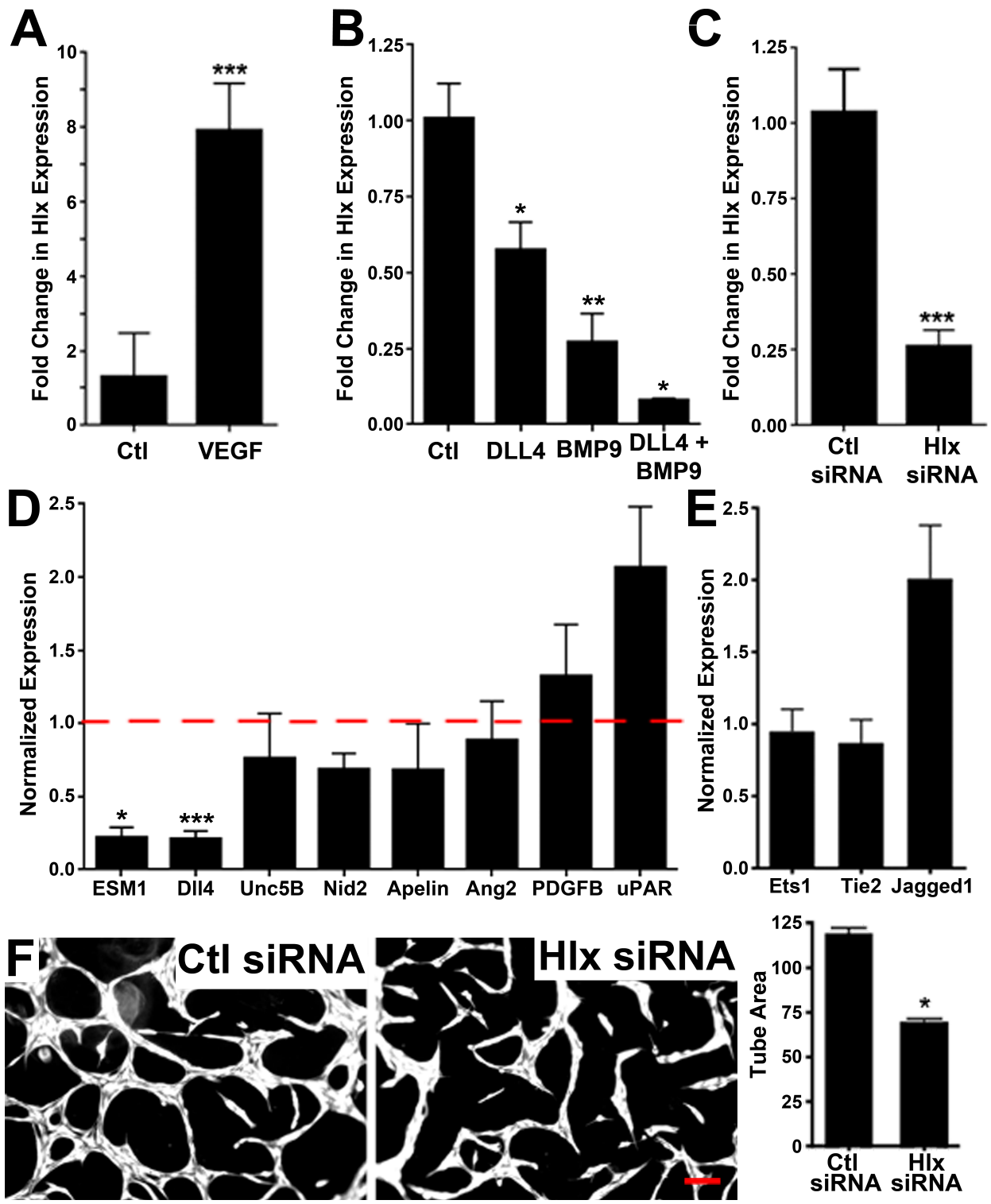
14s
B
20s
hlx1
C
8s
14s
20s
hlx1
D
hlx1 sense
E
7s 14s 20s 4d
Hlx
Hlx Expression
60
50
40
30
20
10
0
**
*
*
7s
14s
20s
4d


A
Fold Change in Hlx Expression
10
8
6
4
2
0
***
Ctl
VEGF
B
Fold Change in Hlx Expression
1.25
1.00
0.75
0.50
0.25
0.00
*
**
*
Ctl
DLL4
BMP9
DLL4 + BMP9
C
Fold Change in Hlx Expression
1.25
1.00
0.75
0.50
0.25
0.00
***
Ctl siRNA
Hlx siRNA
D
Normalized Expression
2.5
2.0
1.5
1.0
0.5
0.0
*
***
ESM1
Dll4
Unc5B
Nid2
Apelin
Ang2
PDGFB
uPAR
E
Normalized Expression
2.5
2.0
1.5
1.0
0.5
0.0
Ets1
Tie2
Jagged1
F
Ctl siRNA
Hlx siRNA
Tube Area
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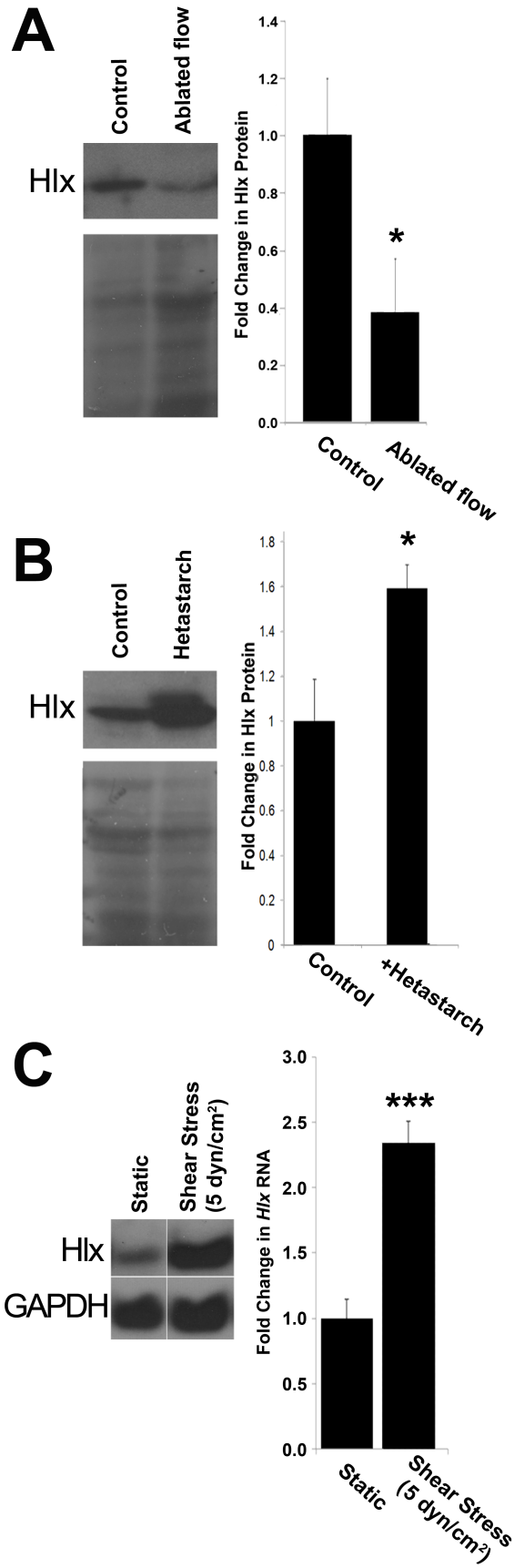
100
75
50
25
0
*
Ctl siRNA
Hlx siRNA


A
Control
Ablated flow
Hlx
Fold Change in Hlx Protein
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1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Ablated flow
B
Control
Hetastarch
Hlx
Fold Change in Hlx Protein
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
+Hetastarch
C
Static
Shear Stress (5 dyn/cm²)
Hlx
GAPDH
Fold Change in Hlx RNA
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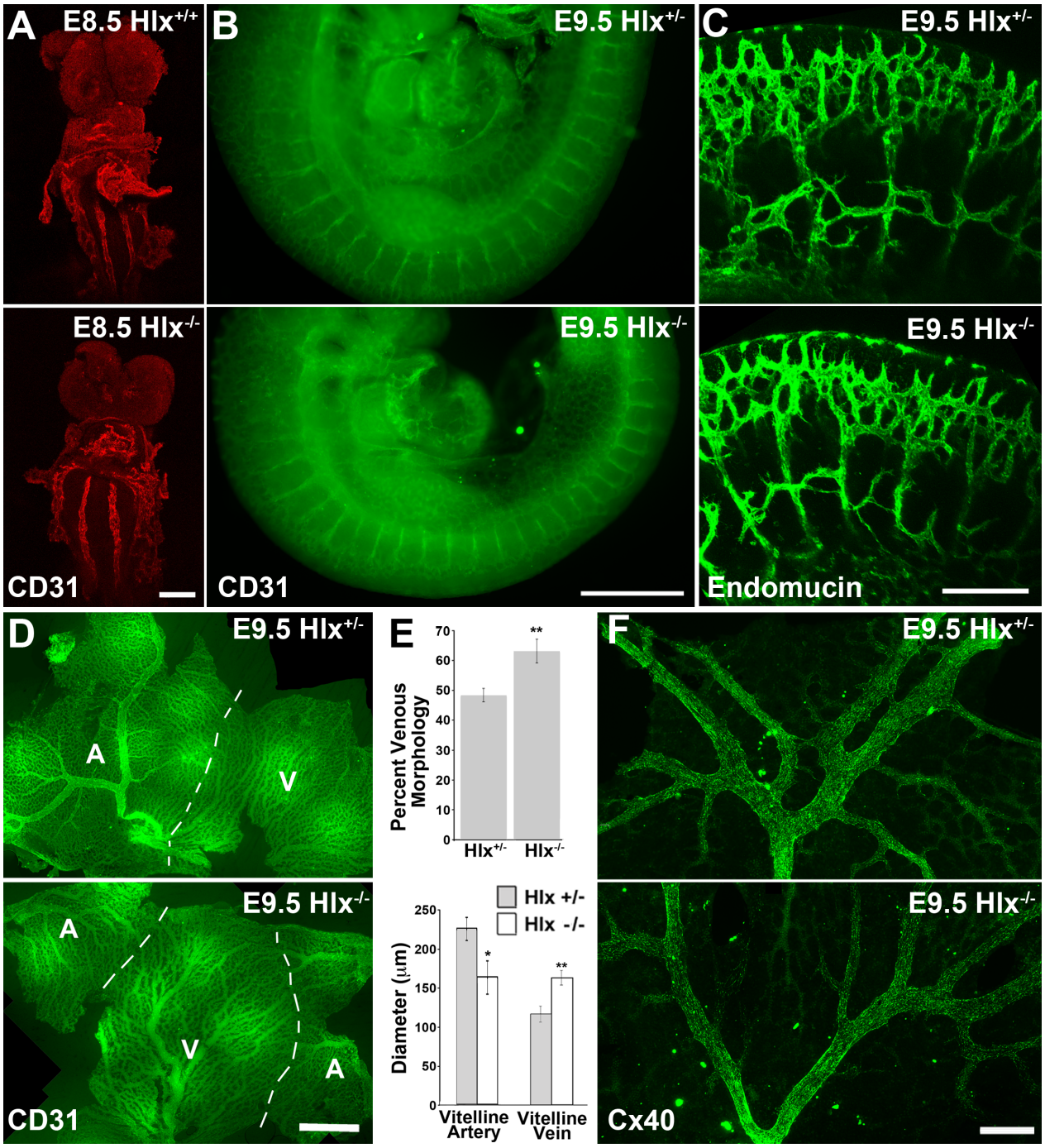
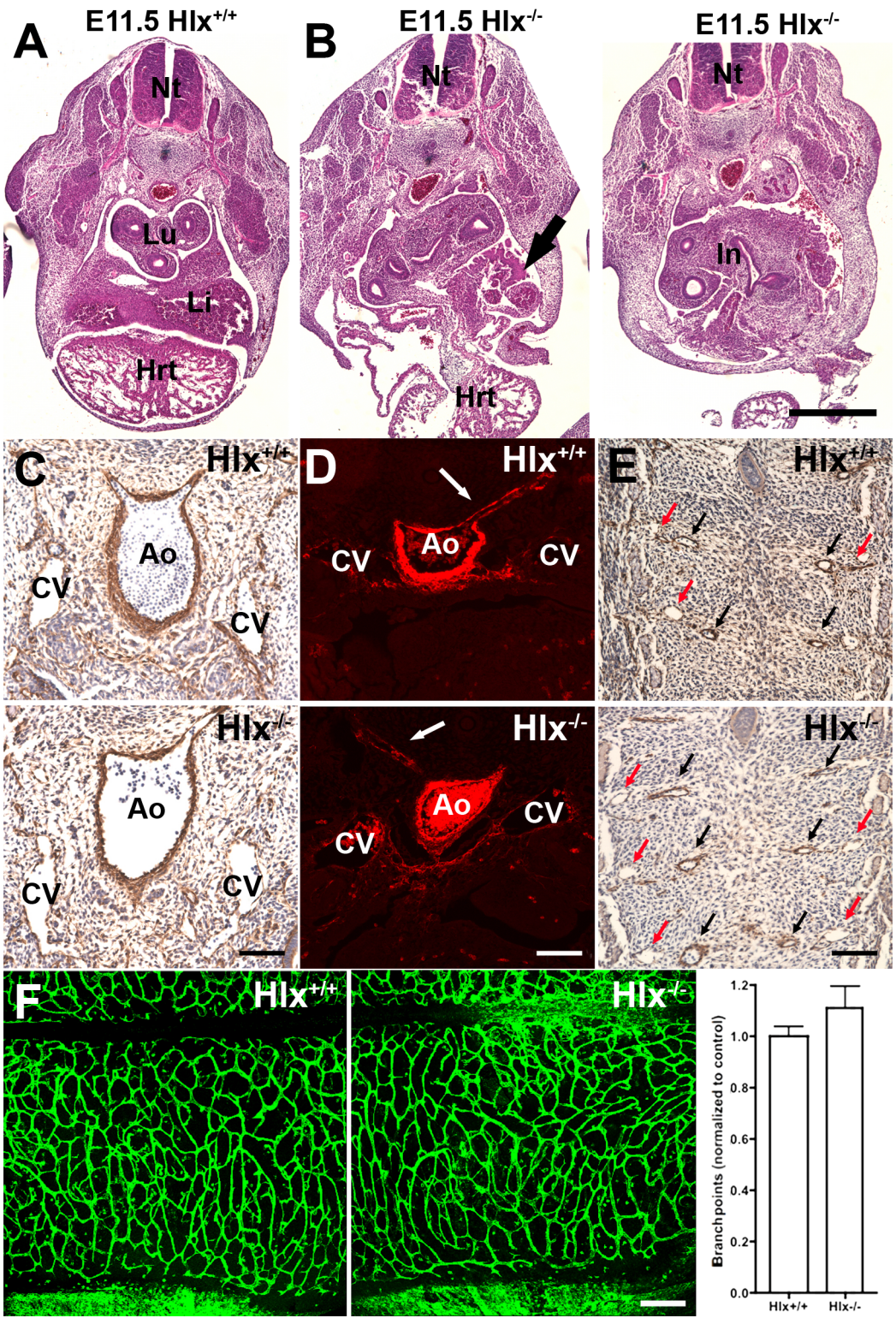
2.5
2.0
1.5
1.0
0.5
0.0
***
Static
Shear Stress (5 dyn/cm²)


A
E8.5 Hlx+/+
B
E9.5 Hlx+/-
C
E9.5 Hlx+/-
E8.5 Hlx-/-
E9.5 Hlx-/-
E9.5 Hlx-/-
CD31
CD31
Endomucin
D
E9.5 Hlx+/-
A
V
E
Percent Venous Morphology
70
60
50
40
30
20
10
0
**
Hlx+/-
Hlx-/-
F
E9.5 Hlx+/-
E9.5 Hlx-/-
A
V
A
CD31
Hlx +/-
Hlx -/-
Diameter (μm)
250
200
150
100
50
0
*
**
Vitelline Artery
Vitelline Vein
Cx40


A
E11.5 Hlx+/+
Nt
Lu
Li
Hrt
B
E11.5 Hlx-/-
Nt
Hrt
E11.5 Hlx-/-
Nt
In
C
Hlx+/+
Ao
CV
CV
D
Hlx+/+
Ao
CV
CV
E
Hlx+/+
Hlx-/-
Ao
CV
CV
Hlx-/-
Ao
CV
CV
Hlx-/-
F
Hlx+/+
Hlx-/-
Branchpoints (normalized to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Hlx+/+
Hlx-/-